Exhibit 3

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# *SHH* Mutation Is Associated With Solitary Median Maxillary Central Incisor: A Study of 13 Patients and Review of the Literature

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**Solitary median maxillary central incisor (SMMCI) or single central incisor is a rare dental anomaly. It has been reported in holoprosencephaly (HPE) cases with severe facial anomalies or as a microform in autosomal dominant HPE (ADHPE). In our review of the literature, we note that SMMCI may also occur as an isolated finding or in association with other systemic abnormalities. These anomalies include short stature, pituitary insufficiency, microcephaly, choanal atresia, midnasal stenosis, and congenital nasal pyriform aperture stenosis. SMMCI can also be a feature of recognized syndromes or associations or a finding in patients with specific chromosomal abnormalities. We performed a molecular study on a cohort of 13 SMMCI patients who did not have HPE. We studied two genes, *Sonic Hedgehog* (*SHH*) and *SIX3*, in which mutations have been reported in patients showing SMMCI as part of the HPE spectrum. A new missense mutation in *SHH* (I111F), segregating in one SMMCI family, was identified. Our results suggest that this mutation may be specific for the SMMCI phenotype since it has not been found in the HPE population or in normal controls.**



KEY WORDS: **solitary median maxillary central incisor (SMMCI); single central incisor; midline defects; HPE; short stature; *Sonic Hedgehog* (*SHH*); *SIX3*; review**

## INTRODUCTION

The congenital absence of one or more teeth in the permanent dentition (oligodontia) has a prevalence of 2.3%–10%. It most frequently involves third molars (25% prevalence) or agenesis of the second premolars or maxillary lateral incisors. In contrast, agenesis of the primary teeth is rare and occurs in less than 1% of the population [Silverman and Ackerman, 1979; Jorgenson, 1980; Winter and Brook, 1986]. It generally affects the incisor region: absence of a primary tooth is followed by agenesis of the succeeding permanent tooth. Specifically, the presence of a solitary median maxillary central incisor (SMMCI) in both primary and permanent dentitions is a rare dental finding.

The mechanisms underlying the congenitally missing maxillary incisor leading to a SMMCI is unknown. It may be due to a congenitally missing bud with agenesis of the incisor and the remaining incisor erupts in the midline [Yassin and El-Tal, 1998]. It has been hypothesized that the formation of one instead of two teeth could result from a disturbance in the mitotic potential of the incisor tooth bud, which could be under genetic and environmental determinants [Osborn and Ten Cate, 1983]. It has also been postulated [Hall et al., 1997] that a critical absence of or reduction in lateral growth from the midline, on or about gestational day 37 or 38, results in premature fusion of the epithelial dental lamina, thus preventing the formation of two complete and separate central incisor teeth. Instead, one tooth consisting of two normal distal halves of the

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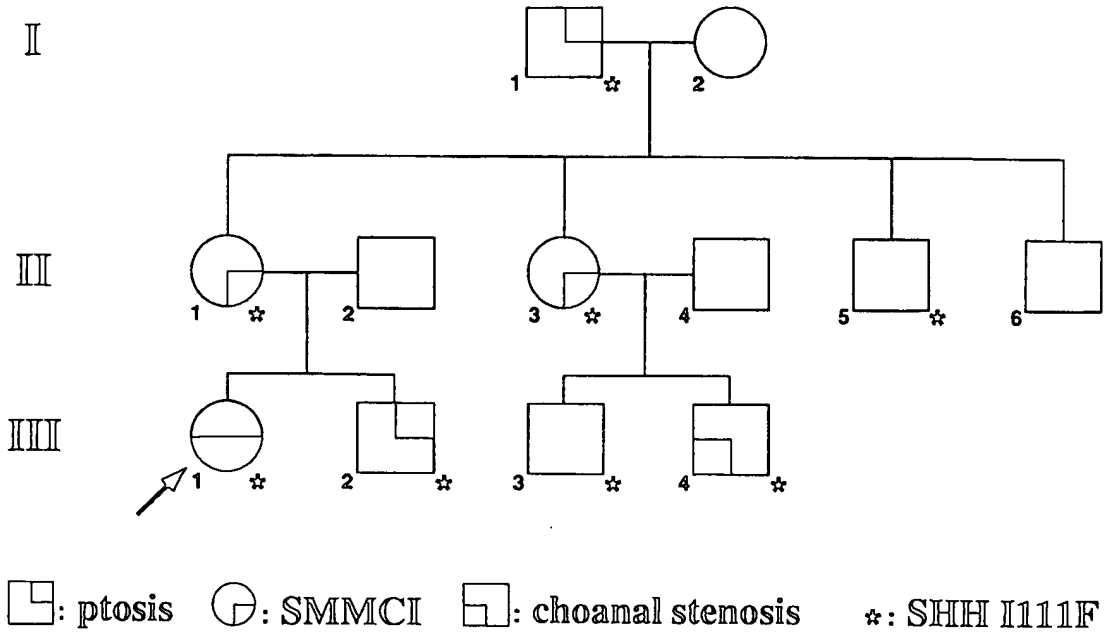



Fig. 1. Pedigree of family with autosomal dominant SMMCI.

central incisors develops from the "fused" enamel knots and subadjacent mesenchyme of these tooth germs.

The SMMCI is unique in that both crown and root are symmetrical, it develops precisely in the midline, and it is the sole central incisor present (with the crown and root size the same as that of a normal central incisor) [Maréchaux, 1986]. In contrast, a mesiodens is a conical, usually asymmetric, permanent series tooth, which, while it may erupt in the midline between two normal central incisor teeth, develops to either the right or left side of the midline. SMMCI was first described as an isolated defect in a 6-year-old girl [Scott, 1958]. Since then, SMMCI has been reported both as an apparently isolated dental finding and with a variety of midline developmental defects, holoprosencephaly, and/or pituitary dysfunction.

Holoprosencephaly (HPE) is a complex developmental field defect of the forebrain in which the cerebral hemispheres fail to separate into distinct halves [for a review, see Golden, 1998; Rubenstein and Beachy, 1998]. Associated craniofacial anomalies can be severe and may include cyclopia, proboscis-like nasal structure, midline cleft palate, and premaxillary agenesis [for a review, see Muenke and Beachy, 2000]. The craniofacial malformations observed in HPE involve the median structures derived from the frontonasal process: interorbital region, nose, prolabium, ethmoidus, nasal bones, nasal septum, and premaxillary bones with the alveolar processes and the four maxillary incisors [Camera et al., 1992]. The phenotypic expression of HPE is quite variable. SMMCI can occur in association with other severe facial anomalies in patients with HPE [Cohen, 1990]. Some individuals with SMMCI, normal intelligence, and normal brain imaging have had children with HPE [Nanni et al., 1999]. Thus, SMMCI has been recognized as a risk factor for holoprosencephalic offspring and may be considered one of the least severe manifestations (microforms) in the spectrum of malformations seen in autosomal dominant HPE (ADHPE) [Lowry, 1974; Berry et al., 1984; Hattori et al., 1987; Fryns and Van den Berghe, 1988; Jaramillo et al., 1988]. Since SMMCI can be part of the HPE spectrum, we investigated whether genes associated with human HPE might also cause isolated SMMCI that is not known to be associated with HPE.

We report a molecular study of 13 unrelated individuals with SMMCI without known HPE or a family history of HPE. We studied two genes associated with HPE, *Sonic Hedgehog* (*SHH*) and *SIX3* [Roessler et al., 1996; Wallis et al., 1999]. A *SHH* (I111F) missense mutation was identified in eight members of a single family: three had a SMMCI; two had ptosis; one was a newborn with choanal stenosis and other defects; and two had a normal phenotype.

## MATERIALS AND METHODS

### Patient Description

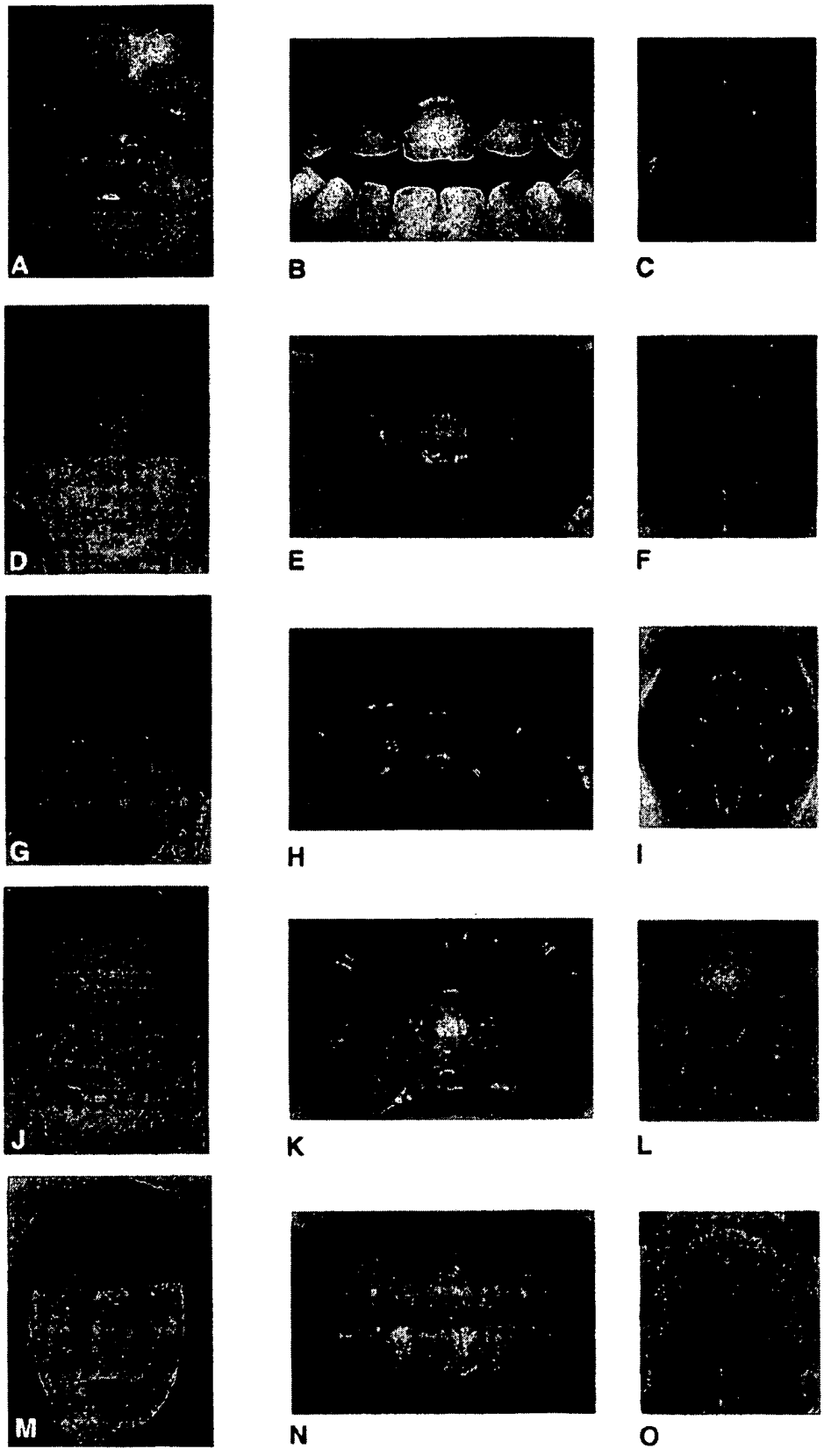
Thirteen unrelated individuals with SMMCI were identified at the Department of Dentistry, Royal Children's Hospital, and the Victorian Clinical Genetics Service, Melbourne, Australia. A study of the extended family of one individual with SMMCI is also part of this report (Fig. 1). Thirty-eight unaffected relatives of the SMMCI cases were also available for study. These individuals were Caucasians, many from an Irish background.

Clinical findings for the 13 unrelated SMMCI cases (6 females and 7 males) are listed in Table I and depicted

TABLE I. Clinical Findings of Individuals With SMMCI in the Present Study

| | Case number[a] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 5 | 6 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 |
| Sex | F | M | M | M | F | F | M | M | M | F | F | M | F | F |
| Short stature (< – 2.0 SD) | | – 3.01 SD | – 1.69 SD | | – 5.05 SD | | – 1.2 SD | – 1.9 SD | – 3.73 SD | | | – 1.43 SD | | |
| Congenital nasal stenosis | MNS/CA | CA | CA | CA | MNS | CA/MNS | CA | MNS | CA/MNS | MNS | MNS | CA | MNS | MNS |
| Prominent midpalatal ridge | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Small head circumference | + | | + | | | | + | + | + | | + | | | |
| Hypotelorism (IP< 3rd C) | ≪ 3rd C | < 3rd C | 3rd C | | 3rd C | | | | ≪ 3rd C | 3rd C | 3rd C | ≪ 3rd C | | |
| Cleft lip/palate | CL | | | | | Bifid uvula | | | | | VPI | | | |
| Cervical/thoracic spinal defects | | | | Hemivertebra | Sacral agenesis | | | Scoliosis | | | | | | |
| Other midline defects | | Micropenis | | | | | | TEF | TEF | Ambiguous genitalia | | | | |
| Cardiac defects | | SM | | SM | | | | Tetralogy of Fallot | Tetralogy of Fallot | Tetralogy of Fallot | | | | |
| Mental retardation/epilepsy | SL | Mild IR | SL | | SL | | IR | SL | IR | | | | | SL |
| Known syndromes/ other anomalies | Oligodontia | Panhypopituitarism | | | CTEV | CHARGE | Ptosis | VACTERL | VACTERL Hypothyroid Absent L kidney | Alopecia Anal fissure | VCFS, del(22) (q 11.2) | | Allergies | Allergies |

[a]Note that cases 18 and 19 are from the same family. All other individuals are unrelated. M, Male; F, Female; SD, standard deviation from the mean; MNS, midnasal stenosis; CA, choanal atresis; IP, interpupillary distance; C, Centile; CL, cleft lip; VPI, velopharyngeal incompetence; SM, systolic murmur; SL, slow learner; IR, intellectual retardation; CTEV, congenital talipes equinovarus; TEF, tracheoesophageal fistula with esophageal atresia; VCFS, velo cardio lacial syndrome. As reported in Hall et al. [1997].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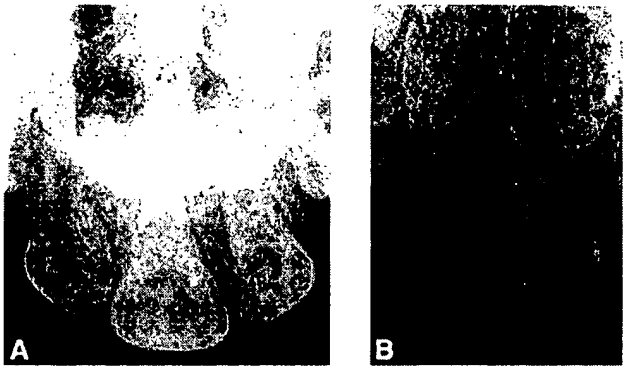


Fig. 3. Radiographs of SMMCI in two unrelated individuals.

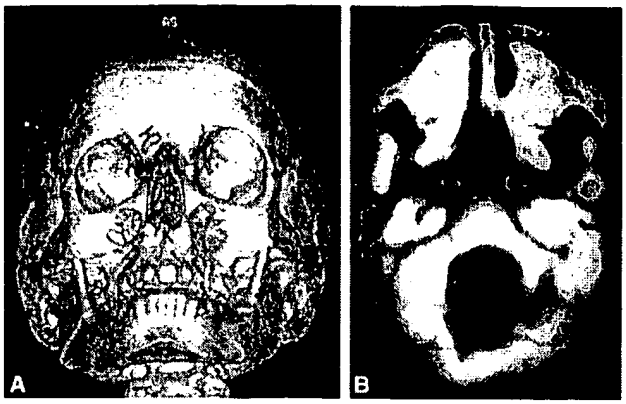


Fig. 4. Skull radiographs of two individuals with SMMCI. **A:** Three-dimensional computer-generated scan of individual 22 (same as J and K in Fig. 2). **B:** CT scan of individual with SMMCI and nasal stenosis on the left side. Numbers of individuals are the same as in Hall et al., 1997.

in Figures 2–4. Individuals 18 and 19 in Table I are from the same family (Fig. 1). Four children had short stature (below – 2.0 SD from the mean for height). One child had panhypopituitarism with an abnormal pituitary fossa and had received treatment with oxandrolone, andriol, thyroxine, hydrocortisone, and sustanon. In all 13 cases, congenital nasal airway stenosis was present. Choanal atresia (Fig. 4) was confirmed in five children, midnasal stenosis was present in six children, and the site of obstruction was uncertain in three other children. All 13 had a prominent midpalatal ridge (Fig. 2). Four SMMCI cases were hypoteloric and had an interpupillary distance below the 3rd centile (Fig. 2). Five children had cardiac defects, including tetralogy of Fallot or systolic murmur. Seven had mental retardation or were slow learners. In four children, SMMCI was part of a known syndrome or association, including VACTERL association (vertebral anomalies, anal atresia, cardiac malformations, tracheo-esophageal fistula with esophageal atresia, and renal and limb anomalies), CHARGE association (coloboma, heart defect, atresia choanae, retarded growth and development, genital anomalies, and ear anomalies), and velocardiofacial syndrome with chromosomal deletion of 22q11. Clinical descriptions have been published previously (Hall et al., 1997).

All samples were obtained by informed consent according to the guidelines of the Victorian Clinical Genetics Service at the Royal Children's Hospital, Melbourne, Australia, and the Children's Hospital of Philadelphia.

### Molecular Studies

Mutation analysis was performed for the entire coding region and exon-intron boundaries of the *SHH* gene using single-strand conformational polymorphism (SSCP) or direct sequencing in 12 individuals. Furthermore, we analyzed the homeodomain of the *SIX3* gene in 11 individuals.

The primer pairs for *SHH* [Roessler et al., 1996, 1997; Nanni et al., 1999] and the homeodomain of *SIX3* [Wallis et al., 1999; Nanni et al., 2000] have been previously published. All PCRs were performed in a PTC-100 thermal cycler (MJ Research, Inc.). SSCP analysis was performed as described elsewhere [Muenke et al., 1994b]. Sequencing of the amplicons demonstrating SSCP band shifts was performed by the Protein and DNA Core Facility of The Children's Hospital of Philadelphia on an ABI Prism$^{TM}$ 377 analyzer.

The *SHH* missense mutation detected by sequencing causes the loss of a MslI restriction site. Restriction fragment length polymorphism (RFLP) analysis was performed as follows: in a 20 μL reaction volume, using 50–70 ng of DNA, 2 μL of 10 × buffer (New England Biolabs 2), and 2 μL of MslI (5,000 U/mL; New England Biolabs), incubated at 37° C for several hours.

## RESULTS

We screened the complete coding region and exon-intron boundaries of the *SHH* gene in 12 DNA samples from unrelated individuals with SMMCI (Table I) and the homeodomain of the *SIX3* gene in 11 DNA samples. None of the SMMCI cases had known HPE or a family history of HPE. A total of three sequence variations was identified: one missense mutation in *SHH* and two putative polymorphisms in *SHH* and *SIX3* (Table II).

The ATC→TTC (I111F) missense mutation in *SHH* was detected in eight members of a SMMCI family (Fig. 1). The proband (III.1) (Table I, case 18), her mother (II.1) (Table I, case 19), and her maternal aunt have a SMMCI. Her brother (III.2) and maternal grandfather (I.1) have ptosis. The proband (III.1) and her maternal cousin (III.4) have midnasal stenosis. Family members with the *SHH* I111F are shown in Figure 1. The

Fig. 2. Facial, dental, and palatal anomalies in individuals with SMMCI. Facial findings are shown in individuals of varying ages in **A, D, G, J,** and **M.** Primary or secondary solitary median maxillary central incisors are depicted in **B, E, H, K,** and **N.** Note the absence of the superior labial frenulum in all individuals with SMMCI; the prominent midline palatal ridge in **C, F, I,** and **O;** and the small nostrils in **L.** A: Individual 3 in Table I. C: Individual 18. D–F: Individual 15. G–I and L: Individual 25. J and K: Individual 22 (same as Fig. 4A). M–O: Individual 19, mother of daughter with SMMCI (same as II.1 and III.1 in Fig. 1). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]. Numbers of individuals are the same as in Hall et al., 1997.

TABLE II. Molecular Studies of HPE Genes in Individuals With SMMCI

| Genes[a] | Nucleotide change | Expected effect | No. of individuals [b] |
|---|---|---|---|
| SHH | ATC→TTC | Ile111Phe | 1 SMMCI family |
| | TCG→TCA | Ser190Ser | 2 unaffected relatives |
| SIX3 | GCG→GCT | Ala240Ala | 7 unaffected relatives |

[a]Complete coding region and exon-intron boundaries for *SHH* gene; homeodomain for *SIX3* gene.
[b]Number of patients carrying that sequence alteration.

sequence change occurs in the N-terminal signaling domain at an invariant position in all of the vertebrate hedgehog proteins. Interestingly, this mutation did not result in a band shift detectable by SSCP, and was only noted after sequencing. The base change was confirmed by the loss of a MslI restriction site. RFLP analysis of 200 HPE chromosomes and 200 control chromosomes did not show this sequence alteration.

One putative polymorphism detected in *SHH* (TCG→TCA) predicts no amino acid change (S190S) (Table II). It was identified in two clinically unaffected relatives of SMMCI case 15 (Table I) in the present study and in two of 225 HPE cases from a different study [Roessler et al., 1997]. Interestingly, one putative polymorphism in *SIX3*, which occurs in the wobble position (GCG→GCT) and does not predict an amino acid change (A240A), was identified in seven unaffected relatives of SMMCI case 15 (six cases) and case 18 (one case), but was not seen in over 200 HPE samples or over 100 normal control samples.

## DISCUSSION

The best described association of SMMCI is as part of the HPE spectrum (Tables III and IV). Clinical expression of HPE is highly variable, ranging from alobar HPE and cyclopia to microforms of HPE even within a single family [Ming and Muenke, 1998]. Mutations in four genes have been associated with HPE: *Sonic Hedgehog* (*SHH*) [Roessler et al., 1996], *ZIC2* [Brown et al., 1998], *SIX3* [Wallis et al., 1999], and *TG-interacting factor* (*TGIF*) [Gripp et al., 2000] Interestingly, mutations in *SHH* or *SIX3* were present in individuals with a SMMCI in familial ADHPE pedigrees. Ten of the 26 mutations detected in *SHH* and 1 of the 4 mutations detected in *SIX3* in the overall HPE population were found in patients with SMMCI as part of the HPE spectrum. Patients with HPE and *ZIC2* mutations do not have severe facial malformations, and SMMCI has not been reported [Brown et al., 1998]. One patient with a *TGIF* mutation had SMMCI [Gripp et al., 2000]. Mice with a homozygous null mutation for *Gli2* have abnormal development of the maxillary incisors [Hardcastle et al., 1998]. It remains to be determined if mutations of this gene occur in humans with SMMCI or HPE.

Since SMMCI can be present as a microform in ADHPE kindreds, we determined if HPE genes are associated with SMMCI in the absence of a known history of HPE. We identified a novel I111F missense mutation in the *SHH* gene in eight individuals in a family showing AD transmission of SMMCI (Fig. 1). This sequence change occurs in a residue conserved in all vertebrate hedgehog proteins and was not found in 200 chromosomes from unrelated HPE cases or 200 chromosomes from unrelated normal individuals by RFLP. Based on these results, we postulate that this *SHH* sequence alteration may be specifically associated with a SMMCI phenotype. However, only functional studies can determine the effect of this sequence change.

Pituitary dysfunction is a major midline developmental defect that can also be associated with SMMCI (Table III). It presents most frequently as isolated growth hormone (GH) deficiency, or it may present with variable degrees of hypopituitarism. Rappaport et al. [1976] introduced the term *monosuperocentroincisivodontic dwarfism* to describe the association between a SMMCI and isolated GH deficiency with concomitant short stature. This association was later confirmed [Vanelli et al., 1980; Artman and Boyden, 1990; Hamilton et al., 1998; Yassin and El-Tal, 1998; Kjellin et al., 1999]. In the series reported by Hall et al. [1997] short stature was found only occasionally. Kjellin et al. [1999] reported a case with congenital pananterior hypopituitarism, carotid aplasia, congenital nasal pyriform aperture stenosis (CNPAS), and SMMCI. They postulated that the vascular anomaly may have induced both hypopituitarism and the single-tooth anomaly.

SMMCI has also been reported in individuals with short stature and normal GH levels (Table III), while other SMMCI patients have normal stature [Wesley et al., 1978; Santoro and Wesley, 1983]. Thus, any SMMCI patient with significant growth retardation should have an evaluation for GH deficiency [Wesley et al., 1978]. SMMCI has also been reported in association with hypothalamic hamartoma and precocious puberty [Winter et al., 1982].

SMMCI is also associated with three types of congenital nasal cavity anomalies: choanal atresia, midnasal stenosis, and CNPAS (Table III). Choanal atresia and/or midnasal stenosis are commonly found in CHARGE association; Antley-Bixler syndrome; Lenz-Majewski hyperostosis syndrome; ectrodactyly, ectodermal dysplasia, clefting (EEC) syndrome; and HPE [reviewed in Hall et al., 1997]. Midnasal stenosis is a bony narrowing of the midnasal cavity, commonly unilateral, characterized by diffuse hypoplasia and seen in a variety of craniofacial syndromes. CNPAS is an anterior nasal cavity obstruction secondary to bony overgrowth of nasal processes of the maxilla. Although CNPAS may be an isolated anomaly, Arlis and Ward [1992] found that four of six such patients had a SMMCI. Moreover, later reports showed CNPAS in association with SMMCI and pituitary hypofunction [Huang et al., 1998; Lo et al., 1998; Kjellin et al., 1999]. Thus, SMMCI and CNPAS may represent parts of a developmental field defect in which a midfacial dysostosis is associated with endocrine and CNS (e.g., HPE) abnormalities.

Mild midline facial defects, such as orbital hypotelorism, broad nasal groove, high-arched narrow palate,

TABLE III. Systemic Abnormalities Reported in Patients With SMMCI Without a Recognized Syndrome

| System/organ | Associated findings | References |
|---|---|---|
| Endocrine system | Short stature (24) | 2, 14, 18, 22, 23, 27, 29, 34, 37, 53, 55, 71, 78 |
| | Growth hormone deficiency (16) | 2, 22, 23, 34, 55, 71, 78 |
| | Hypoplastic pituitary/(pan)hypopituitarism (6) | 2, 22, 29, 34, 38, 43 |
| | Thyroid dysgenesis (1) | 1 |
| | Hypoglycemia (1) | 43 |
| | Jaundice (1) | 43 |
| | Precocious puberty (1) | 75 |
| | Grave disease (1) | 55 |
| CNS | Holoprosencephaly (28) | 6, 10, 11, 12, 17, 26, 31, 33, 39, 47, 49, 51, 67, 73 |
| | Microcephaly (9) | 5, 18, 21, 22, 65, 78 |
| | Agenesis corpus callosum (2) | 21, 70 |
| | Hypoplastic/missing left cochlea (2) | 62, 65 |
| | Hypothalamic hamartoma (1) | 77 |
| | J-shape sella turcica (1) | 55 |
| | Spina bifida (1) | 14 |
| | Seizures/nystagmus/spastic contractures (1) | 65 |
| | Epilepsy (1) | 22 |
| | Rhinorrhea CS fluid (1) | 65 |
| | Gross cerebral dysmorphism (1) | 65 |
| Cardiovascular system | Ventricular septal defect (1) | 15 |
| | Complex vascular anomaly (absent ICA) (1) | 34 |
| | Fallot Tetralogy of (1) | 22 |
| | Systolic murmur (2) | 22 |
| | Aberrant subclavian artery (1) | 22 |
| | Persistent ductus Botalli (1) | 14 |
| | Congenital heart defect (1) | 18 |
| | Cyanotic attacks (1) | 14 |
| Gastrointestinal tract | Ectopic anus (1) | 14 |
| | Tracheo-esophageal fistula/esophageal atresia (2) | 22 |
| | Anal fistula (1) | 22 |
| Eyes | Hypotelorism (18) | 1, 22, 33, 35, 55, 65, 71, 72 |
| | Iris coloboma (4) | 5, 22, 37, 44 |
| | Slanting palpebral fissures (1) | 65 |
| | Microphthalmia/unreactive pupils (2) | 2, 65 |
| | Staphylomas (1) | 37 |
| | Convergent strabismus (1) | 22 |
| | Ptosis (1) | 22 |
| | Visual defects (1) | 14 |
| | Congenital cataracts (1) | 20 |
| Nose | Choanal atresia/midanasal stenosis (18) | 14, 22 |
| | CNPAS (15) | 1, 21, 29, 34, 38, 59, 72 |
| | Depressed/flat nasal bridge (4) | 5, 55, 78 |
| | Deviated nasal septum (1) | 78 |
| | Anteverted tip/nostrils (4) | 55, 62, 74 |
| | Hypoplastic nose (4) | 1, 65 |
| | Broad nasal groove (2) | 55, 74 |
| | Broad nose (1) | 55 |
| Ears | Malformed ear lobes (1) | 65 |
| Oral cavity | Prominent midpalatal ridge (torus palatinus) (21) | 5, 22, 33 |
| | Narrow high-arched palate (10) | 1, 33, 35, 55, 65, 71, 72 |
| | Prominent philtrum (2) | 62, 74 |
| | Absence philtrum contours upper lip (3) | 33 |
| | Cleft lip/palate (3) | 22 |
| | Submucous cleft plate (1) | 65 |
| | Bifid/hypoplastic uvula (2) | 22, 65 |
| Skin | Incontinentia pigmenti achromians (1) | 5 |
| | Alopecia (1) | 22 |
| | Multiple hemangiomata (1) | 22 |
| Genitalia | Cryptorchism (1) | 78 |
| | Micropenis (1) | 22 |
| | Ambiguous genitalia (1) | 22 |

TABLE III. (*Continued*)

| System/organ | Associated findings | References |
|---|---|---|
| Skeletal system/limb | Klippel-Feil deformity (1) | 15 |
| | Cervical hemivertebra (1) | 22 |
| | Cervical dermoid (1) | 22 |
| | C2-C3 spinal process fusion (1) | 5 |
| | Anomalies cervical vertebras/sacral agenesis (1) | 22 |
| | Scoliosis (1) | 18 |
| | Hypoplasia I metacarpal (1) | 65 |
| | Congenital talipes equinovarus (2) | 22 |
| | Partial syndactyly 3-4 toes (1) | 55 |
| | Absent thumb (1) | 22 |
| Mental status | Slow learning abilities (5) | 22, 78 |
| Apparently isolated SMMCI | (15) | 4, 28, 30, 36, 40, 41, 63, 66 |

[1]Arlis and Ward, 1992; [2]Artman and Boyden, 1990; [4]Bamba, 1989; [5]Bartholomew et al., 1987; [8]Berry et al., 1984; [10]Camera et al., 1992; [11]Cohen, 1990; [12]Collins et al., 1993; [14]Ellisdon and Marshall, 1970; [15]Fleming et al., 1990; [17]Fryns and Van den Berghe, 1988; [18]Fulstow, 1968; [20]Gorlin et al., 2001; [21]Gripp et al., 2000; [22]Hall et al., 1997; [23]Hamilton et al., 1998; [26]Hattori et al., 1987; [27]Hayward, 1979; [28]Holm and Lundberg, 1972; [29]Huang et al., 1998; [30]Hunter et al., 1991; [31]Jaramillo et al., 1988; [33]Kjaer et al., 1997; [34]Kjellin et al., 1999; [35]Kocsis, 1994; [36]Kopp, 1967; [37]Liberfarb et al., 1987; [38]Lo et al., 1998; [39]Lowry, 1974; [40]Marechaux, 1986; [41]Mass and Sarnat, 1991; [43]Matthai and Smith, 1996; [44]Ming and Muenke, 1998; [47]Muenke et al., 1994a; [49]Nanni et al., 1999; [51]Odent et al., 1999; [53]Parker and Vann, 1985; [55]Rappaport et al., 1977; [58]Royal et al., 1999; [62]Santoro and Wesley, 1983; [63]Scott, 1958; [65]Simon and Roberts, 1993; [66]Small, 1979; [67]Süβ et al., 1990; [70]Thesleff et al., 1995; [71]Vanelli et al., 1980; [72]Walker et al., 1996; [73]Wallis et al., 1999; [74]Wesley et al., 1978; [77]Winter et al., 1982; [78]Yassin and El-Tal, 1998.

and median cleft palate, have been reported (Table III). Additional findings have been associated in a patient with SMMCI, ventricular septal defect, Klippel-Feil deformity with hemivertebra, and extra rib [Fleming et al., 1990].

SMMCI has rarely been reported as part of syndromes or associations with more severe midline anomalies, including VACTERL association [Wesley et al., 1978; Hall et al., 1997], CHARGE association [Hall et al., 1997; Harrison et al., 1997], and velocardiofacial syndrome [Hall et al., 1997] (Table IV). SMMCI was also reported in a patient with hypomelanosis of Ito, iris coloboma, microcephaly, developmental delay, and ventriculomegaly by CT [Bartholomew et al., 1987]. Moreover, SMMCI has been associated with autosomal dominant and recessive ectodermal dysplasia [Winter et al., 1988; Buntinx and Baraitser, 1989].

Lastly, SMMCI has also occasionally been associated with chromosomal abnormalities (Table IV). The 18p deletion is associated with HPE in 10% of cases and has also been reported in association with SMMCI in four cases (without HPE). A ring chromosome 18 in a patient with CNPAS, SMMCI, premaxillary dysgenesis, and GH deficiency has also been reported [Tavin et al., 1994]. SMMCI with 7q terminal deletions (7q32→qter) have been reported in four cases [Masuno et al., 1990; Frints et al., 1998]. A single case with SMMCI and deletion of 22q11.2 has been reported [Hall et al., 1997]. A patient with 47,XXX also had SMMCI [Miura et al., 1993]. Not surprisingly, several of these deletions are in chromosomal regions that harbor HPE genes [Roessler and Muenke, 1998].

It is likely that a number of mechanisms can give rise to a SMMCI, and some may also cause HPE. At present, the risk of HPE in the offspring of an individual with SMMCI is unclear. Determining the genetic basis of SMMCI should provide a greater understanding of the mechanisms underlying the genesis of the SMMCI and its clinical significance.

TABLE IV. Known Syndromes, Associations and Chromosomes Abnormalities Reported in Patients With SMMCI

| | References |
|---|---|
| Syndromes/associations | |
| CHARGE association (3) | 22, 25 |
| VACTERL association (3) | 22, 74 |
| Velocardiofacial syndrome [del(22)(q11.2)] (1) | 22 |
| Ectodermal dysplasia (AD/AR) (2) | 9, 76 |
| Autosomal dominant HPE (25) | 6, 10, 11, 12, 17, 26, 31, 39, 47, 49, 51, 67, 73 |
| Chromosomal abnormalities | |
| del(18p) (4) | 3, 7, 13, 68 |
| r(18) (1) | 69 |
| del(7)(q36→qter) (4) | 16, 42 |
| 47,XXX (1) | 45 |

[3]Aughton et al., 1991; [6]Berry et al., 1984; [7]Boudailliez et al., 1983; [9]Buntinx and Baraitser, 1989; [10]Camera et al., 1992; [11]Cohen, 1990; [12]Collins et al., 1993; [13]Dolan et al., 1981; [16]Frints et al., 1998; [17]Fryns and Van den Berghe, 1988; [22]Hall et al., 1997; [25]Harrison et al., 1997; [26]Hattori et al., 1987; [31]Jaramillo et al., 1988; [39]Lowry, 1974; [42]Masuno et al., 1990; [45]Miura et al., 1993; [47]Muenke et al., 1994a; [49]Nanni et al., 1999; [51]Odent et al., 1999; [97]Süβ et al., 1990; [68]Taine et al., 1997; [69]Tavin et al., 1994; [73]Wallis et al., 1999; [74]Wesley et al., 1978; [76]Winter et al., 1988.

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Exhibit 4



letter

# Mutations in *IHH*, encoding Indian hedgehog, cause brachydactyly type A-1

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**Brachydactyly type A-1 (BDA-1; MIM 112500) is characterized by shortening or missing of the middle phalanges (Fig. 1*a*)[1]. It was first identified by Farabee in 1903 (ref. 2), is the first recorded example of a human anomaly with Mendelian autosomal-dominant inheritance and, as such, is cited in most genetic and biological textbooks. Here we show that mutations in *IHH*, which encodes Indian hedgehog, cause BDA-1. We have identified three heterozygous missense mutations in the region encoding the amino-terminal signaling domain in all affected members of three large, unrelated families. The three mutant amino acids, which are conserved across all vertebrates and invertebrates studied so far, are predicted to be adjacent on the surface of IHH.**

In 1951, Bell categorized five types of inherited brachydactyly (BD) on the basis of malformation of the digits: A, B, C, D and E[1]. Type A is divided into three subtypes—A1, A2 and A3—according to the classifications of Bell[1] and Fitch[3]. Since then, progress has been made in understanding the aetiology of different types of BD[4–6]. We recently mapped the locus for BDA-1 in two large unrelated families (families I and II, Fig. 1*c*) to an 8.1- cM interval on chromosome 2q35–q36 flanked by markers *D2S2248* and *D2S360*, and excluded the possibility that mutations in *PAX3* cause the disorder[7]. We decided to focus on *IHH*, composed of 3 exons and spanning 5.5 kb of genomic DNA (Fig. 2*a*), owing to its position in the implicated interval and because it is known to mediate condensation, growth and differentiation of cartilage[8].

To screen for possible mutations in *IHH*, we designed primers according to the sequence of mouse *Ihh* mRNA because the sequence data of full-length human *IHH* was unavailable. We designed primers to amplify the coding regions, promoter region (2 kb), and splice junctions of genomic DNA, and screened affected and unaffected family members and control individuals. Affected individuals in family I have a G→A transition at position 283 of exon 1 (Fig. 3*a*), which is predicted to effect a Glu95→Lys substitution, and affected individuals in family II have a G→A transition at position 391 of exon 2 (Fig. 3*b*), which is predicted to result in a Glu131→Lys substitution. We screened a third pedigree with eight unaffected and five affected members showing typical BDA-1 (family III, Fig. 1*c*). Analysis by X-ray shows that the hand bones of affected members (Fig. 1*a*) have the

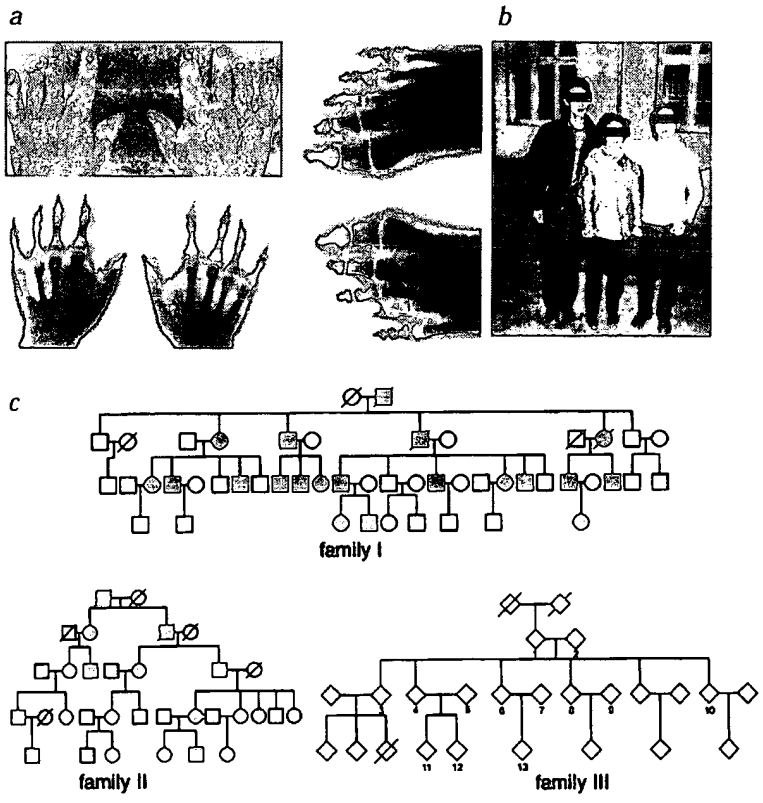


**Fig. 1** Phenotype of BDA-1 and pedigree structure. ***a***, The hands and feet of an affected individual in family III. In some affected individuals all middle phalanges are missing or fused to the distal phalanges; in others, one to three fingers or toes have missing or fused phalanges. ***b***, Affected individual with two children (the affected child is on the right) in family III. Note short stature. ***c***, BDA-1 pedigrees I, II and III; affected individuals are denoted by filled symbols.

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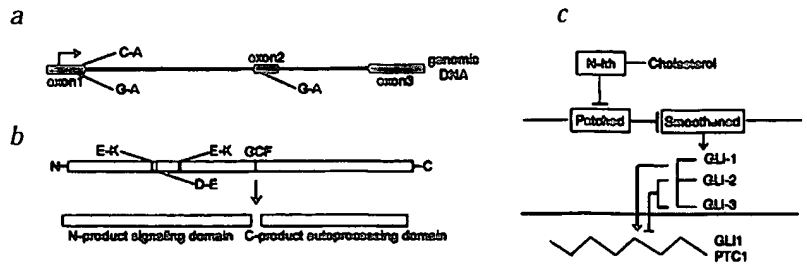


**Fig. 2** Structure of IHH and function of SHH. *a*, Structure of *IHH* and location of mutations. *b*, Schematic of IHH protein and sites of amino acid residue transition and cleavage. *c*, Signalling pathway of SHH. Upon cleavage, the amino terminal fragment (N-hh) binds cholesterol, enabling it to bind its receptor, Patched. This abrogates the inhibitory effect of Patched on Smoothened, leading to activatation of downstream Gli transcription factors.

same morphology as those in families I and II. Furthermore, affected members are shorter (1.50–1.60 m) than unaffected individuals (1.65–1.80 m) in family III (Fig. 1*b*). All affected individuals in this family have a C→A transversion at position 300 of exon 1 (Fig. 3*c*), which is predicted to result in an Asp100→Glu substitution.

Restriction endonuclease digestion confirms the mutations in the three families (Fig. 3*d–f*). All show complete segregation with disease and are not observed in unaffected members of the families. They are also not observed in unrelated BuYi Chinese (100 chromosomes), Miao Chinese (100 chromosomes), and Han Chinese (392 chromosomes). Familes I, II and II are BuYi Chinese, Miao Chinese and Han Chinese, respectively.

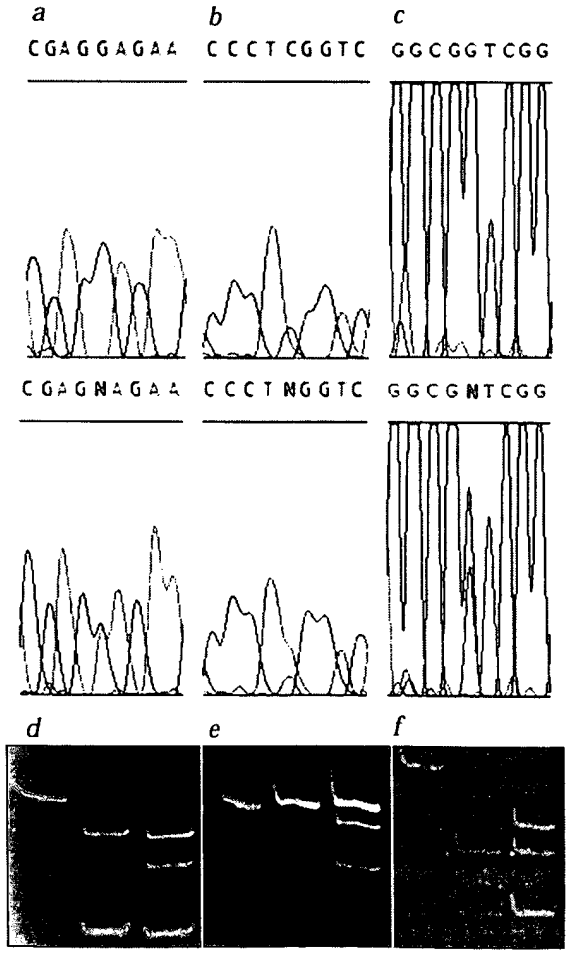


**Fig. 3** Mutation analysis. Top, normal sequence; middle, heterozygous mutant sequence (site of mutation is indicated by boldface N); bottom, confirmation of mutations by digestion with endonuclease. *a*, G283→A mutation in family I. *b*, G391→A mutation in family II (reverse sequence). *c*, C300→A mutation in family III (reverse sequence). *d*, G283→A mutation creates a new *MboII* site. *e*, G391→A mutation creates a *StyI* site. *f*, C300→A mutation deletes an *AciI* site. Bands represent PCR products, PCR digestion products of unaffected individuals and PCR digestion products of affected individuals from left to right in *d*, *e* and *f*, respectively.

Cross-species alignment indicates that the IHH mutant amino acids (in addition to those in SHH that cause holoprosencephaly[9]) are conserved across human, mouse, chicken, African clawed frog, Zebrafish, Japanese common newt, *Drosophila hydei* and *Drosophila melanogaster*.

IHH, sonic hedgehog (SHH) and desert hedgehog (DHH) comprise a conserved signaling family in vertebrates and some invertebrates[10]. The protein products in the hedgehog family are synthesized as precursors that are subsequently autoprocessed by the carboxy-terminal domain to generate a liberated 'amino-terminal' domain responsible for local and long-range signaling activities (Fig. 2*b*)[11]. It would seem that the action and regulation of hedgehog signaling proteins during limb development in vertebrates and invertebrates is similar[12]. The locus implicated in syndactyly type 1 encompasses *IHH*[13]; our findings make *IHH* a stronger candidate for the gene whose mutation underlies this form of syndactyly.

Because of the high similarity between human IHH and mouse Shh, we used the crystal structure of the N terminus of mouse Shh[14] to compare the locations of the implicated amino acids. Glu95, Asp100 and Glu131 are predicted to be in close proximity and on the surface of a groove in SHH (Fig. 4), assuming that the mouse and human orthologs encode proteins of similar stucture. It may be that Glu95→Lys, Asp100→Glu or Glu131→Lys effect aberrant signalling by interfering with SHH binding to its 'natural' receptor(s), or promoting its binding with other receptors. It is claimed[15,16] that some other mutant amino acids in the conserved region around the possible groove have a slight effect on its binding to Patched, a hedgehog receptor[17–19]. It is also possible that BDA-1 is caused by haploinsufficiency of the wildtype protein.

In humans, genetic disruption of the components of the SHH-signalling pathway (Fig. 2*c*) leads to a range of developmental defects[20]. Compared with the effects of SHH-mediated signalling, those of IHH-dependent signaling are less clear. Loss-of-function studies in the mouse demonstrate that Ihh is essential for chondrocyte proliferation. Embryonic mice lacking exon 1 of *Ihh* have foreshortened forelimbs and their digits remain unsegmented and uncalcified[21]. And overexpression of Hip (a hedgehog receptor that modulates hedgehog signaling) in cartilage leads to a similarly shortened skeleton[22]. The small stature of the affected individuals in family III indicates that further study of this family may provide additional insight into the mechanisms of IHH signaling.

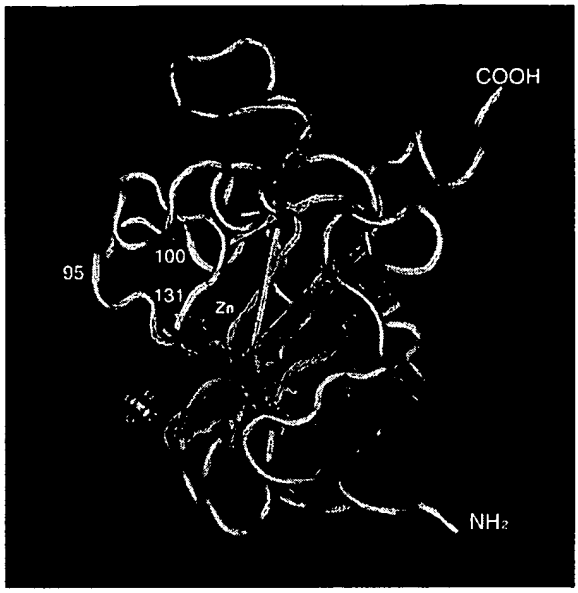


**Fig. 4** Three-dimensional analysis based on mouse Shh. The three amino acids (95, 100 and 131) in yellow are at adjacent locations on the surface of the crystal structure of murine Shh. Green and brown indicate α-helix and β-strand, respectively. The small tetrahedron represents a sulfate ion.

## Methods

**Patients.** We recruited two large families, one BuYi Chinese (family I) from Hunan Province, China, and one Miao Chinese (family II) from GuiZhou Province, China, as previously described[3]. We studied an additional family (family III) in Hong Jiang, Hunan Province, China, in this work. Subjects are identified by number, as marked under the symbols in the pedigrees. All participants gave informed consent.

In individuals 1 and 10 of family III, the middle phalanges of digits 2, 3 and 4 are missing (they are fused to the distal phalange). Similarly, individuals 4 and 11 lack the middle phalanges of digits 2, 4 and 5. Individual 11 is missing the middle phalange of digit 3 (it is fused to the proximal phalange). Individual 4 has a middle phalange of digit 3, but it is shorter than usual. Individual 6 has only the middle phalange of digit 5 missing (it is fused to the distal phalange); her/his other digits are present but are shorter than usual.

**Mutation analysis.** We took samples of peripheral blood DNA from all available family members. We isolated DNA by standard procedure and carried out polymerase chain reaction (PCR) analysis using primers designed to amplify coding sequence, splice junctions and the promoter region. We sequenced the PCR products.

**Amplification of *IHH*.** We used primers 1F (5′-CGGACGCTATGAAG GCAAGA-3′) and 1R (5′-GCCAGCCAGTCGAGAAAATG-3′) to amplify genomic DNA that is part of exon 1 and primers 2F (5′-GCGCCTACAC CTGCACCTC-3′) and 2R (5′-CCTTCTCGGCACTACTCCTCCT-3′) to amplify genomic DNA containing exon 2 by the touchdown PCR program with additional Q-solution from Qiagen. We electrophoresed the PCR products on 1.5% agarose gels. The primers were selected with either Primer Premier (version 5.0) or an on-line program PRIMER3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and were synthesized by Bioasia. We carried out amplification by PCR using an MJ Research RT-225 Peltier thermal cycler.

**Sequencing of *IHH*.** We used a kit (Promega wizard) to purify PCR products. We sequenced both strands using the PCR primers and the ABI Prism BigDye terminator cycle sequencing reaction kit (PE Applied Biosystems). We analyzed the products with an ABI 377 DNA sequencer or an ABI 3100 DNA sequencer.

**Mutation confirmation.** As the mutation in family I created a new *Mbo*II site, the mutation in family II created a *Sty*I site and the mutation in family III deleted an *Aci*I site, digestions of the 1F-1R PCR product and 2F-2R PCR product were carried out under standard conditions. We separated the products on a 20% polyacrylamide gel.

**Three-dimensional structure analysis.** We analyzed the three-dimensional structure with Cn3D version 3.0 from NCBI with the three-dimensional crystal structure of an N-terminal fragment of mouse Shh.

**GenBank accession numbers.** Human *IHH* mRNA, L38517; mouse *Ihh* mRNA, U85610; human hedgehog gene, exon1, AB010581.

**PDB accession ID.** mouse sonic hedgehog, N-terminal domain structure, 1VHH.

**Swiss-Prot accession numbers.** Human IHH precursor, Q14623; mouse Ihh precursor, P97812; mouse Shh precursor, Q62226; human SHH precursor, Q15465.

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